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Use of BHK_{21}-adapted mesogenic Newcastle disease virus for primary vaccination of chicks

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ABSTRACT

The use of BHK_{21}-adapted mesogenic 'Komarov' strain of Newcastle disease virus for primary vaccination of chicks was studied. The efficacy of the vaccine was assessed by haemagglutination-inhibiting antibody titres and challenge experiments. The BHK_{21}-adapted virus was safe and potent for priming the chicks by oculonasal route. Further studies are in progress.

BHK_{21} (Razi) cells are known for their ability to grow in suspension. Padmaraj *et al.*(1991) and Kumanan *et al.* (1992) reported that velogenic NDV isolates grow up to high titres in BHK_{21} cells as compared to those of low virulence. In the present study the efficacy of BHK_{21} (Razi)-adapted 'Komarov' strain of NDV in primary vaccination of chicks has been described.

MATERIALS AND METHODS

The mesogenic 'Komarov' and lentogenic 'F' strains of NDV were obtained from the Institute of Veterinary Preventive Medicine, Ranipet. The velogenic strain 'NDV VP 12' available at this laboratory was used as the challenge virus.

The 'Komarov' strain of NDV was serially passaged up to 35 passages in BHK_{21} cells maintained in the Central Tissue Culture Laboratory of this Department. The tissue-culture infectivity titres ($TCID_{50}$) and haemagglutination (HA) titres were assessed at the end of every fifth passage. The virulence characters like mean death time (MDT) and intra-cerebral pathogenicity index (ICPI) were assessed after 35 passages (Allan *et al.* 1978).

The virus harvest at 35th passage was used in the vaccination trial. Three groups of White Leghorn chicks each comprising 40 chicks were used. Group 1 received the regular RDVF vaccine on day 7 by oculonasal route. Group 2 received 100 $TCID_{50}$ of BHK_{21}-adapted 'Komarov' virus on day 7 by oculonasal route. Group 3 served as the unvaccinated controls.

The chicks were bled prior to vaccination and thereafter at weekly intervals up to 8 weeks of age. Seroconversion of the vaccinates was assessed by haemagglutination-inhibition test and protection by challenge experiments. From each group 10 birds were challenged at 3 and 6 weeks post-vaccination (PV) with 100 chick-infective dose (CID_{50}) of velogenic NDV. The post-challenge titres were estimated 14 days after both the challenges.

RESULTS AND DISCUSSION

The 'Komarov' strain of NDV adapted well to the BHK_{21} cells. As the passage level increased, the infectivity titre increased gradually, whereas haemagglutination (HA) titre dropped (Table 1). The drop in the HA titre concurred with the earlier findings of Rossi and Acciari (1969) and could be attributed

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Table 1. HA and tissue culture infectivity titres of BHK$_{21}$-adapted 'Komarov' strain

	Passage level							
	0	5	10	15	20	25	30	35
HA titre	512	16	8	4	4	4	4	4
Infectivity titre $\log_{10}$ $TCID_{50}$ per 50µl	-	2.5	3.7	5.2	6.2	6.7	7.7	7.78

to the loss of haemagglutinins during passage in BHK$_{21}$ cells. However, recently Reddy and Srinivasan (1992) reported a rise in both infectivity and HA titre on serial passages in BHK$_{21}$ cells. This warrents further work in establishing the reasons for the rise in the HA titre. After 35 passages the MDT and ICPI values of the virus were 96 hr and 0.28 respectively. The adapted virus had become more a lentogenic strain than mesogenic.

Chicken-embryo-adapted 'F' and 'K' strains are being widely used for primary and secondary vaccination of chicks at the age of 5-7 days and 8 weeks respectively. In an alternative system for NDV vaccine production the tissue-culture-adapted 'K' strain was used for priming the chicks. No untoward reactions were observed when chicks vaccinated with 100 $TCID_{50}$ of BHK$_{21}$ adapted 'K'. The chicks responded well to the BHK$_{21}$-adapted 'K' virus and the HI antibody titres were comparable with those of the RDVF vaccinates (Table 2). There was no significant difference ($P<0.05$) between the 2 vaccines used.

The results of the challenge experiments correlated well with the HI antibody titres with 80 and 50% survivals in BHK$_{21}$-adapted 'K' virus vaccination when challenged on 21 days and 42 days post-vaccination respectively (Table 3).

The protectivity of this vaccine could be increased by increasing the dose to 10 times, i.e. 1000 $TCID_{50}$ instead of the 100 $TCID_{50}$

Table 2. Haemagglutination-inhibiting antibody titres* after vaccination with RDVF and BHK$_{21}$ adapted 'Komarov' strain

On the day of vaccination/ Days post-vaccination	Unvaccinated	BHK$_{21}$-adapted 'Komarov'	RDVF
	5	5	5
7	4.8	5.2	6.1
14	3.6	6.8	6.4
21	2.4	6.4	6.6
28	2.0	5.2	5.8
35	0	5.2	5.2
42	0	4.3	4.6
49	0	3.4	3.2
56		2.4	2.2

*Mean antibody titres expressed as log to base 2.

Table 3. Result of challenge experiments

Groups	21 days post-vaccination			42 days post-vaccination		
	No. survived/challenged	% survived	Post-challenge HI titres	No. survived/challenged	% survived	Post-challenge HI titres
Unvaccinated controls	2*/10	20	4.8	0/10	0	-
BHK_{21} adapted 'Komarov'	8/10	80	9.2	5/10	50	6.4
RDVF	9/10	90	9.4	6/10	60	6.6

* Developed characteristic signs of NDV infection.

used in this experiment. The results were encouraging and concurred with the observations of Reddy and Srinivasan (1992) who used the BHK_{21}-adapted 'Komarov' strain for inducing immunity in 14-week-old chicks.

It therefore appears that the BHK_{21}-adapted 'Komarov' strain of NDV could be used for primary vaccination of chicks without any side effects, thus eliminating the problems of embryo-adapted NDV vaccines. Virus recovery studies with the vaccinates, characterization of the recovered virus as well as studies using 1000 $TCID_{50}$ as the vaccine dose are in progress besides studies to determine the appropriate age of vaccination.

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Newcastle Disease Virus as an Antineoplastic Agent: Induction of Tumor Necrosis Factor-α and Augmentation of Its Cytotoxicity[1,2,3]

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The oncolytic strain 73-T of Newcastle disease virus (NDV) has been reported to be beneficial in the treatment of cancer patients, but little is known about its mechanism of action. In this study, NDV strain 73-T and a wild-type isolate of NDV were found to be potent inducers of tumor necrosis factor (TNF) production by both human peripheral blood mononuclear cells (PBMCs) and rat splenocytes. Antibody inhibition experiments identified TNF-α as the major species of TNF induced by NDV in PBMCs. The effect of recombinant human TNF-α (rHuTNF-α) on human cancer cells was then examined. Neither rHuTNF-α nor supernatants from NDV-stimulated PBMCs were cytotoxic toward the TNF-resistant human malignant melanoma cell line MEL-14. However, when MEL-14 cells were treated with NDV strain 73-T, both rHuTNF-α and supernatants from NDV-stimulated PBMCs killed 48% and 55%, respectively, of these tumor cells. Treatment with NDV also conferred TNF susceptibility to the TNF-resistant human malignant melanoma cell line MEL-21 and the human myelogenous leukemia cell line K562. In contrast to its enhanced cytotoxicity toward NDV-treated cancer cells, rHuTNF-α had no effect on NDV-treated normal human PBMCs proliferating in response to concanavalin A. These results suggest two important mechanisms for the antineoplastic activity of NDV: (*a*) induction of TNF-α secretion by human PBMCs and (*b*) enhancement of the sensitivity of neoplastic cells to the cytolytic effects of TNF-α. [J Natl Cancer Inst 1988;80:1305–1312]

The clinical literature shows that certain viruses can destroy tumors in cancer patients (*1–5*). Such observations led to the initial wave of interest between the years 1950 and 1971 in treating cancer patients with viruses (*6–8*). However, a major drawback to this type of therapy was the observed neurotropism of the most effective oncolytic viruses, which caused encephalitis in some patients.

In an attempt to eliminate this side effect, Cassel and Garrett (*9*) used the oncolytic strain 73-T of Newcastle disease virus (NDV), which was isolated after 73 passages in vitro and 13 passages in vivo in mouse Ehrlich ascites tumor cells. This paramyxovirus caused no side effects when it was injected at high doses into humans (*9,10*). NDV strain 73-T killed some human cancer cells in vitro, caused necrosis of tumors in hamsters, and could effect a cure of mouse ascites tumors after they reached 41% of their total development (*9*). In clinical trials on humans, NDV strain 73-T replicated in the tumor of a patient with cervical cancer and reduced the size of the tumor (*9*). More recently, Cassel and co-workers (*11,12*) showed that of 32 patients with stage II malignant melanoma who were treated with an NDV oncolysate (consisting of a concentrate of live virus in tumor cell membranes), 90% remained disease free after 3 years, in contrast to <10% of control patients. In experiments with mice having a lymphoma, Heicappell et al. (*13*) and Schirrmacher et al. (*14*) recently verified that NDV and NDV-modified tumor cells can cause tumor regression and can prevent metastases.

Recent reports (*15,16*) have indicated that another paramyxovirus, Sendai virus, is a potent inducer of the monocyte/macrophage product, tumor necrosis factor (TNF), in human peripheral blood mononuclear cells (PBMCs). In light of these observations, we postulated that NDV strain 73-T would also induce secretion of TNF. TNF, also called cachectin (reviewed in refs. *17,18*), was first described by Carswell et al. (*19*) as an antitumor substance released into the sera of BCG-sensitized mice after injection with endotoxin. Since this macrophage product shares many properties with lymphotoxin, a product of mitogen- or antigen-activated lymphocytes, it has been renamed TNF-α and the lymphocyte product has been renamed TNF-β. The activities of these two cytokines include the ability to mediate cytotoxic or cytostatic effects on certain tumor lines in vitro and hemorrhagic necrosis of some tumors in vivo (*20–24*). TNF-α and TNF-β share 30% amino acid homology (*25,26*), interact at the same cell-surface receptor (*27,28*), and have genes linked to the major histocompatibility complex (*29*).

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[3]This article is dedicated to the memory of Donald Bouthillier.

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Regarding the potential use of TNF as a therapeutic agent in humans, recombinant TNF-α has been recently tested in phase I trials (*30*). Single doses of up to 200 μg of recombinant human TNF-α (rHuTNF-α)/m^2 of body-surface area were well tolerated in cancer patients (*30*). However, in animal models, the therapeutic action of TNF-α has been limited by its toxicity. Asher et al. (*31*) observed that tumor-bearing mice were more susceptible to the toxic side effects of rHuTNF-α than were normal mice. Havell et al. (*32*) found that almost lethal quantities of recombinant murine TNF-α (rMuTNF-α) were required to cause tumor regression in mice. Observations of such toxic properties are consistent with the proposed role of TNF-α in cachexia (*17*) and endotoxic shock (*33*).

Endogenous production of TNF-α at tumor sites may be one way to limit the toxic effects of TNF-α. In this article, we show that NDV strain 73-T, which has already been shown to replicate in human and murine tumors (*9*), not only is a potent inducer of TNF-α in human PBMCs but also confers sensitivity to TNF-α in human tumor cell lines that are normally resistant to the lytic effects of TNF-α. These observations indicate a role for endogenous TNF-α in the antineoplastic properties of NDV in cancer patients.

Materials and Methods

Viruses

NDV strain 73-T was obtained from Dr. William A. Cassel, Emory University, Atlanta, GA. A wild-type isolate of NDV was a gift from Dr. Deoki N. Tripathy, University of Illinois, Urbana, IL. These strains of NDV were grown in the allantoic cavity fluid of 11-day fertilized chicken eggs, harvested after 2 days, and quantified in hemagglutination units (HAU) (*34*).

Tumor Cells

The human myelogenous leukemia cell line K562 was provided by Dr. David Kranz, University of Illinois, Urbana, IL. The human malignant melanoma cell lines MEL-14 and MEL-21 were gifts from Dr. Michael J. Walker and Dr. Tapas Das Gupta, University of Illinois, Chicago, IL. The murine fibrosarcoma cell line WEHI 164 (clone 13) was obtained from Dr. Terje Espevik, Genentech, Inc., South San Francisco, CA. The murine transformed fibroblast line L929 (ATCC CCL 1) was provided by the American Type Culture Collection, Rockville, MD.

Recombinant TNF and Antibodies

The following products were gifts from Dr. H. Michael Shepard of Genentech, Inc.: rHuTNF-α, 5.0×10^7 U/mg; recombinant human TNF-β (rHuTNF-β), 1.2×10^8 U/mg; rMuTNF-α, 2.9×10^7 U/mg; affinity-purified monoclonal antibody against rHuTNF-α from mouse ascites, 6.000 neutralizing units/μg of protein; and affinity-purified rabbit antiserum to rHuTNF-β, 2.9×10^7 neutralizing units/mL. One unit of TNF has been defined as the reciprocal of the dilution of a preparation that results in 50% killing of dactinomycin (Act D)-treated L929 cells (*35*).

Interferons

Interferons (IFNs) that were tested in the TNF assay were purified cell-derived murine IFN-α (Lee Biomolecular, San Diego, CA) and recombinant rat IFN-γ(Amgen, Thousand Oaks, CA).

TNF Assay

TNF was detected by a bioassay according to the procedure of Espevik and Nissen-Meyer (*36*), with minor modifications. WEHI 164 clone 13 target cells were seeded in 96-well microplates (Falcon Microtest III plates; Becton, Dickinson & Co., Oxnard, CA) at a concentration of 4×10^4 cells/well in 100 μL of medium RPMI-1640 (Sigma Chemical Co., St. Louis, MO) containing 10% heat-inactivated fetal bovine serum (FBS), 100 U of penicillin/mL, and 100 μg of streptomycin/mL. After the cells were incubated overnight at 39 °C in 7% CO_2 in a humidified chamber, the medium was removed and replaced with 50 μL/well of RPMI-1640 containing 10% FBS and 1.33 μg of Act D/mL (Sigma Chemical Co.). Test samples of 50 μL in RPMI-1640 containing 10% FBS were then added to each well. After the cells were incubated for an additional 18 hours, a cytotoxicity assay was performed with the use of the organic dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma Chemical Co.) according to the methods of Mosmann (*37*), with minor modifications. The medium was removed from each well and replaced with 50 μL of 1 mg of MTT/mL in 0.85% saline. After the cells were incubated at 39 °C for 4 hours, the MTT-containing solution was removed (while not disturbing any of the blue tetrazolium crystals within the cells) and replaced with 50 μL of 0.015 *M* phosphate-buffered saline (PBS) (pH 7.2) and 100 μL of acid-isopropanol (0.04 *N* HCl in isopropanol) per well. After the blue crystals were dissolved by repeated pipetting, the absorbance at 540 nm was determined by using a Bio-Tek model 310 enzyme-linked immunosorbent assay reader (Bio-Tek Instruments Inc., Burlington, VT). Cells that were lysed with 0.01% Triton X-100 (TX-100) (Sigma Chemical Co.) served as a measurement of 100% cytotoxicity. Percent specific cytotoxicity due to a sample containing TNF was determined by the formula: percent specific cytotoxicity = $100 \times$ {[absorbance (cells + medium) − absorbance (cells + sample)]/[absorbance (cells + medium) − absorbance (cells + TX-100)]}.

Human PBMC and Rat Splenocyte Preparations

Six milliliters of heparinized (100 U/mL) whole blood, collected from healthy male volunteers between 25 and 35 years of age, was added to 6.7 mL of Sepracell-MN (Sepratech Corp., Oklahoma City, OK). After being mixed by gentle inversion, the tubes were centrifuged at 2,000 *g* at room temperature. The PBMC layer was removed and washed once with 15 mL of 0.015 *M* PBS containing 0.1% bovine serum albumin (BSA), hereafter referred to as PBS/BSA, at pH 7.2. Cells were pelleted by centrifugation at 300 *g* for 10 minutes at room temperature. Erythrocytes in the PBMC pellet were lysed by resuspending the pellet in 0.5 mL of 0.83% NH_4Cl in 0.1% $KHCO_3$–0.01 m*M* EDTA at 4 °C for 3 min-

utes. PBMCs were washed an additional three times with PBS/BSA and resuspended at 10^6 cells/mL in RPMI-1640 without serum.

Wistar-Furth rats were housed and cared for in accordance with University of Illinois guidelines. Single-cell suspensions of splenocytes were prepared from 3-month-old female rats (*38*). Erythrocytes were lysed as described above, and splenocytes were resuspended in RPMI-1640 without serum.

TNF Induction

PBMCs, (10^6 cells/mL) and rat splenocytes (3×10^6 cells/mL) were incubated for 24 hours at 39 °C in 7% CO_2 with NDV and control egg allantoic fluid at various concentrations. Supernatants were obtained by centrifuging the medium at 4,000 *g* at 4 °C for 5 minutes. Supernatants were then heated at 60 °C for 30 minutes to kill any remaining virus, as described by Berent et al. (*16*), before dilution in RPMI-1640 and assaying for TNF. Such heat inactivation of the virus does not affect TNF activity (*16*).

Cytotoxicity Assay of Human Cancer Cells

The nonadherent K562 cells were incubated for 3 hours at a concentration of 4×10^6 cells/mL in RPMI-1640 containing 10% FBS with 100 μCi of ^{51}Cr (Amersham Corp., Arlington Heights, IL). Labeled cells were then washed three times with 20 mL of RPMI-1640 before resuspension at 4×10^5 cells/mL in RPMI-1640 containing 10% FBS. One hundred microliters of this cell suspension was added to each well in a 96-well microtiter plate. Cells in each well were treated by addition of medium, NDV, or control egg allantoic fluid diluted in 50 μL of RPMI-1640 containing 10% FBS. After 2 hours of incubation with NDV, TNF samples in 50 μL of RPMI-1640 containing 10% FBS were added to each well and incubated for 18 hours. After the microtiter plate was centrifuged at 400 *g* for 10 minutes at room temperature, 100 μL of supernatant was removed from each well, mixed with 3 mL of Aquasol-2 liquid scintillation cocktail (DuPont/NEN Products, Boston, MA), and counted in a Beckman LS-5801 scintillation counter. Cells were treated with 0.1% TX-100 to determine maximal release of ^{51}Cr. Percent specific cytotoxicity was calculated from the following formula: percent specific cytotoxicity = 100 × [(sample counts per minute − spontaneous counts per minute)/(maximum release counts per minute − spontaneous counts per minute)].

Adherent MEL-14 and MEL-21 cells were plated in a 96-well microtiter plate at a concentration of 4×10^4 cells/well in 100 μL of Hanks' balanced salt solution (Sigma Chemical Co.), 10% FBS, and 1 μCi of ^{51}Cr. After the cells were incubated overnight at 39 °C, radioactive medium was removed and each well was washed three times with 200 μL of RPMI-1640 before addition of 100 μL of medium and 50 μL of NDV or control medium. After a 2-hour treatment period, control TNF samples or supernatants (100 μL) to be tested for TNF were added to each well. Following an incubation of 18 hours at 39 °C with 7% CO_2, 100 μL of medium from each well was counted and the percent cytotoxicity was determined as above. Spontaneous release of ^{51}Cr never exceeded 25% of the maximum release for any of the target cells that were used in these experiments. Evidence that K562, MEL-14, and MEL-21 cells were indeed infected with NDV was based on cytopathic changes that were observed microscopically in some cells 3 days after addition of NDV.

Effects of NDV and TNF on Lectin-Induced Proliferation of PBMCs

Cytotoxic effects of NDV and TNF on human PBMCs proliferating in response to 5 μg of concanavalin A (Con A)/mL were measured by use of the ^{51}Cr procedures described for the nonadherent K562 cells.

The cytostatic effects of NDV and TNF on human PBMCs proliferating in response to Con A were also determined. PBMCs at 5×10^6 cells/mL were added in 100-μL aliquots to microtiter wells. Two days after the addition of 100 μL of Con A (final concentration of 5 μg/mL), cells were incubated with or without 10 HAU of NDV and 1 μCi of [*methyl*-^{3}H]thymidine (6.7 Ci/mmol). Two hours later, aliquots of various TNF concentrations were added, and the samples were incubated for an additional 16 hours. Cells were then harvested onto glass-fiber disks and dried. Finally, 3 mL of a toluene-omnifluor (DuPont/NEN) scintillation cocktail was added, and radioactivity was measured in a Beckman LS 5801 liquid scintillation counter.

Antibody Inhibition Studies

Specific antibodies (23 neutralizing units) against either rHuTNF-α, rHuTNF-β, or control medium without antibodies were mixed with either 1 U of rHuTNF-α, 1 U of rHuTNF-β, or 10 μL of supernatant from NDV-stimulated human PBMCs. After incubation at 39 °C for 2 hours, samples (in 30 μL) were tested for TNF activity in the TNF assay.

Results

Sensitivity of WEHI 164 Clone 13 Cells to Picogram Quantities of rHuTNF-α and rHuTNF-β

In table 1 a standard method of measuring cytotoxicity using ^{51}Cr release of labeled WEHI 164 clone 13 targets was compared with a more recent and sensitive cytotoxicity assay (*37*) based on viability determination using the dye MTT. Both assays yielded similar results (table 1). Treatment of WEHI 164 clone 13 cells with the RNA-synthesis inhibitor Act D resulted in a slight increase in the percent cytotoxicity caused by either rHuTNF-α or rHuTNF-β. This effect is well illustrated with 1 U of rHuTNF-α, which had a 37% specific cytotoxicity toward WEHI 164 clone 13 cells in the absence of Act D and a 73% specific cytotoxicity in its presence. Similar results were obtained with rHuTNF-β. Under the various assay conditions tested (table 1), WEHI 164 clone 13 cells were sensitive to 0.1 U of TNF, which corresponded to 2 pg of rHuTNF-α and 0.8 pg of rHuTNF-β. These results are similar to those of Espevik and Nissen-Meyer (*36*).

Table 1. Sensitivity of murine fibrosarcoma WEHI 164 clone 13 cells to cytotoxic effects of rHuTNF-α and rHuTNF-β*

Amount of TNF (U)	% specific cytotoxicity				
	rHuTNF-α			rHuTNF-β	
	^{51}Cr release†	MTT without Act D‡	MTT with Act D‡	MTT without Act D	MTT with Act D
0	0 ± 1	0 ± 4	0 ± 4	0 ± 2	0 ± 7
0.01	6 ± 1	1 ± 1	1 ± 5	7 ± 8	6 ± 9
0.1	32 ± 3	34 ± 5	49 ± 3	28 ± 1	43 ± 3
1	48 ± 1	37 ± 4	73 ± 3	57 ± 1	91 ± 2
10	69 ± 6	78 ± 2	99 ± 1	90 ± 1	91 ± 2
25	89 ± 4	ND	ND	ND	ND
100	ND	97 ± 1	103 ± 1	100 ± 1	100 ± 1

*Values = means ± SEM of at least three different assays. ND = not determined.

†Percent specific cytotoxicity was measured by ^{51}Cr release of labeled cells in an 8-hr assay.

‡Percent specific cytotoxicity was measured by using an MTT-cell viability assay after cells were incubated with TNF samples for 18 hr with or without 0.67 μg of Act D/mL.

Lack of Cytotoxicity of Cell-Derived Murine IFN-α and Recombinant Rat IFN-γ Toward WEHI 164 Clone 13 Cells

While rHuTNF-α, rHuTNF-β, and rMuTNF-α were all highly toxic toward WEHI 164 clone 13 cells, the IFNs that were tested at much higher concentrations (murine IFN-α and recombinant rat IFN-γ) had negligible cytotoxicity toward these target cells (table 2). Therefore, these cytokines are not expected to interfere in the detection of TNF-α or TNF-β with the use of the WEHI 164 clone 13 cells.

Cytotoxicity of Heat-Inactivated Supernatants of NDV-Treated Human PBMCs and NDV-Treated Rat Splenocytes Toward WEHI 164 Clone 13 Cells and Transformed Murine L929 Fibroblasts

As sections B and C of table 3 show, heat-inactivated supernatants from NDV-stimulated human PBMCs and from NDV-stimulated rat splenocytes were cytotoxic toward WEHI 164 clone 13 cells. For example, supernatants (1:5,000 dilution) from NDV strain 73-T-stimulated human PBMCs (10^6 cells/mL) killed 48% of the WEHI 164 clone 13 cells. Supernatants (1:667 dilutions) from NDV strain 73-T-stimulated rat splenocytes (10^6 cells/mL) killed 100% of the WEHI 164 clone 13 cells. Supernatants (1:667 dilution) from rat splenocytes (10^6 cells/mL) stimulated with wild-type NDV also induced significant cytotoxicity (49%) in these target cells.

To exclude the possibility that some of the cytotoxic activity in the supernatants was due to direct effects of NDV on the target cells, all supernatants were heated at 60 °C for 30 minutes. TNF remains stable under these conditions (*16*). As section D of table 3 shows, heat-inactivated NDV had no direct cytotoxic effect on WEHI 164 clone 13 cells.

Although L929 cells cultured in the presence of Act D are commonly used as a bioassay for TNF activity, L929 cells cultured without Act D were much less sensitive to TNF compared with the sensitivity of WEHI 164 clone 13 cells (table 3, section A). For example, 1 U of rHuTNF-α killed 77% of the WEHI 164 clone 13 cells, while it had no cytotoxic effect on L929 cells cultivated without Act D. Therefore, by using these two cell lines, we were able to determine whether NDV induces accumulation of other toxic products, such as reactive oxygen intermediates, from PBMCs and rat splenocytes that might nonspecifically kill cells. A 1:5,000 dilution of supernatants from NDV-treated human PBMCs and a 1:667 dilution of NDV-treated rat

Table 2. Lack of cytotoxity of cell-derived murine IFN-α and recombinant rat IFN-γ toward murine fibrosarcoma WEHI 164 clone 13 cells

Cytokine	Amount (pg)	% specific cytotoxicity*
rHuTNF-α	100	99 ± 1
rHuTNF-β	100	91 ± 2
rMuTNF-α	100	90 ± 3
Recombinant rat IFN-γ	5,000	1 ± 3
Murine IFN-α	7,000	3 ± 5

*Values = means ± SEM of three assays. Percent specific cytotoxicity was measured by using an MTT-cell viability assay after cells were incubated with cytokine samples for 18 hr with 0.67 μg of Act D/mL.

Table 3. Cytotoxicity of heat-inactivated supernatants of NDV-treated human PBMCs and NDV-treated rat splenocytes toward murine fibrosarcoma WEHI 164 clone 13 cells and murine transformed L929 fibroblasts*

Treatment	% specific cytotoxicity	
	WEHI 164 clone 13†	L929‡
A. rHuTNF-α (U)		
0	0 ± 3	0 ± 5
1	77 ± 3	2 ± 4
10	96 ± 1	7 ± 4
100	100 ± 1	12 ± 5
B. Supernatants from human PBMCs treated with§		
Medium	7 ± 6	1 ± 5
400 HAU of NDV strain 73-T	48 ± 5	4 ± 1
Control egg fluid	2 ± 5	5 ± 2
C. Supernatants from rat splenocytes treated with§		
Medium	5 ± 3	1 ± 1
200 HAU of NDV strain 73-T	100 ± 2	6 ± 2
500 HAU of NDV strain 73-T	99 ± 2	6 ± 2
200 HAU of wild-type NDV	49 ± 3	3 ± 1
Control egg fluid	2 ± 9	3 ± 2
D. Control medium treated with¶		
Medium	0 ± 5	0 ± 1
500 HAU of NDV strain 73-T	3 ± 6	1 ± 3
500 HAU of wild-type NDV	3 ± 5	1 ± 6

*Values = means ± SEM of three assays (section A), supernatants from three human volunteers (section B) or three rats (section C), and three control cultures (section D).

†Percent specific cytotoxicity was measured by using an MTT-cell viability assay with 0.67 μg of Act D/mL.

‡Percent specific cytotoxicity was measured by using an MTT-cell viability assay without Act D.

§All supernatants were heated at 60 °C for 30 min and added at a dilution of 1:5,000 (human PBMCs) or 1:667 (rat splenocytes) to the WEHI 164 clone 13 cells. Both human and rat supernatants were used at a dilution of 1:667 in the assay on L929 cells.

¶Control medium (RPMI-1640 with 10% FBS) was treated with NDV, heated at 60 °C for 30 min, and added at a dilution of 1:667 to both types of target cells.

splenocytes were cytotoxic toward murine WEHI 164 clone 13 cells but not toward proliferating murine L929 cells. These results support the conclusion that NDV induces both human PBMCs and rat splenocytes to secrete TNF, and this protein, rather than other toxic products, is responsible for killing the target WEHI 164 clone 13 cells.

Based on the assumption of a linear relationship between percent cytotoxicity and the amount of rHuTNF between 0.1 and 10 U (tables 1, 3), 419 U and 295 U of TNF were estimated to be produced by 10^6 human PBMCs and 10^6 rat splenocytes, respectively, upon stimulation with NDV (table 4). Production of TNF resulting from control egg allantoic fluid was negligible.

Identification by Specific Antibodies of TNF-α as Major Species of TNF Induced by NDV in Human PBMCs

As table 5 shows, an affinity-purified monoclonal antibody against rHuTNF-α inhibited 91% of the activity of rHuTNF-α, whereas it did not affect the activity of rHuTNF-β. Similarly, an affinity-purified polyclonal antibody against rHuTNF-β inhibited 99% of the activity of rHuTNF-β, but it did not affect the activity of rHuTNF-α.

These specific antibodies against the two species of TNF were used to investigate TNF activity induced by NDV in human PBMC supernatants. Nearly all of the activity in supernatants from NDV-treated human PBMCs was inhibited by antibodies against rHuTNF-α (98% inhibition), whereas only a small amount of activity was inhibited by antibodies against rHuTNF-β (12% inhibition; table 5). These experiments identified TNF-α as the major species of TNF induced by NDV in human PBMCs.

Sensitivity to rHuTNF-α Conferred by Treatment of TNF-Resistant Human Cancer Cells With NDV

Untreated human malignant melanoma MEL-14 cells were totally resistant up to 2,500 U of rHuTNF-α (table 6). However, upon exposure to NDV strain 73-T, the MEL-14 cells became remarkably sensitive to killing by TNF. One hundred units and 2,500 U of rHuTNF-α killed 21% and 48%, respectively, of the NDV-infected MEL-14 cells. Similarly, heat-inactivated supernatants from NDV-treated human PBMCs displayed enhanced cytotoxic effects toward the NDV-infected MEL-14 cells (55% vs. 8%). NDV alone had no cytotoxic effect on these cancer cells. This finding supports the conclusion that TNF-α was present in the supernatants from NDV-treated human PBMCs.

When another human malignant melanoma cell line (MEL-21) was used, rHuTNF-α had no effect on the untreated cells (table 7). NDV alone killed 17% of the MEL-21 cells, but addition of 100 U of rHuTNF-α enhanced this cytotoxic effect fourfold (67%). Less dramatic but significant results were noted for human myelogenous leukemia K562 cells (table 7). Again, rHuTNF-α was not cytotoxic toward the untreated cells. NDV alone killed 20% of the cells, and this cytotoxicity was enhanced almost twofold upon addition of rHuTNF-α to the NDV-treated cells (34%). TNF-α was

Table 5. Use of specific antibodies against rHuTNF-α and rHuTNF-β to identify TNF-α as major species of TNF induced by NDV in human PBMCs

Treatment	% specific cytotoxicity toward WEHI 164 clone 13 cells		
	No antibody	Anti-rHuTNF-α	Anti-rHuTNF-β
rHuTNF-α, 1 U	82 ± 1	7 ± 1 (91)	90 ± 1 (−10)
rHuTNF-β, 1 U	47 ± 1	53 ± 1 (−12)	1 ± 1 (99)
Supernatant from NDV-stimulated human PBMCs (1:125 dilution)	98 ± 1	2 ± 2 (98)	86 ± 2 (12)

*Values = means ± SEM for three assays, each with rHuTNF-α and rHuTNF-β and supernatants of PBMCs from two human volunteers. Percent inhibition is given in parentheses. Percent specific cytotoxicity was measured by using an MTT-cell viability assay with 0.67 μg of Act D/mL. Antibody inhibition experiments were performed as described in Materials and Methods section.

Table 4. Estimation of amount of TNF induced in human PBMCs and rat splenocytes by NDV strain 73-T

Cells	Treatment	TNF production (U/10^6 cells)*
Human PBMCs	Medium	46 ± 41
	Control egg fluid	<30
	NDV strain 73-T‡	419 ± 47
Rat splenocytes	Medium	<30
	Control egg fluid	<30
	NDV strain 73-T‡	295 ± 2

*Values = means ± SEM of supernatants of cells from three human volunteers or three rats. Procedure for estimation of TNF production is described in Materials and Methods section.

†Four hundred hemagglutination units of NDV strain 73-T added per 10^6 cells.

‡One hundred and sixty-seven hemagglutination units of NDV strain 73-T added per 10^6 cells.

Table 6. Cytotoxicity of rHuTNF-α and supernatants from NDV-stimulated human PBMCs toward NDV-infected but not toward uninfected malignant melanoma MEL-14 cells*

NDV infection of MEL-14 cells	Treatment	% specific cytotoxicity toward MEL-14 cells†
−	Medium	0 ± 1
−	rHuTNF-α, 100 U	0 ± 3
−	rHuTNF-α, 2,500 U	4 ± 1
−	NDV-stimulated PBMC supernatant	8 ± 4
+	Medium	0 ± 1
+	rHuTNF-α, 100 U	21 ± 5
+	rHuTNF-α, 2,500 U	48 ± 7
+	NDV-stimulated PBMC supernatant	55 ± 7

*Values = means ± SEM of three assays of supernatants of PBMCs that were uninfected (−) or infected (+) with 10 HAU of NDV. PBMCs were obtained from two human volunteers. All supernatants were heat treated at 60 °C for 30 min before being added to ^{51}Cr-labeled MEL-14 cells.

†Percent specific cytotoxicity was determined after 18 hr of treatment with the use of a ^{51}Cr release assay described in Materials and Methods section.

Table 7. Effects of rHuTNF-α and NDV treatment on human malignant melanoma MEL-21 cells, human myelogenous leukemia K562 cells, and human PBMCs proliferating in response to Con A

NDV added (HAU)	rHuTNF-α (U)	% specific cytotoxicity*		
		MEL-21	K562	PBMCs
0	0	0 ± 8	0 ± 1	0 ± 0
0	100	3 ± 6	2 ± 1	4 ± 4
10	0	17 ± 4	20 ± 1	3 ± 2
10	100	67 ± 1	34 ± 1	2 ± 2

*Values = means ± SEM of two different assays on each type of cell. Percent specific cytotoxicity was determined after 18 hr of treatment with the use of a ^{51}Cr assay described in Materials and Methods section.

demonstrated to be the cytolytic molecule, because antibodies against rHuTNF-α blocked 100% of the cytolytic activity of rHuTNF-α against NDV-treated tumor cells.[6]

Sensitivity to Lytic Effects of TNF-α Not Induced by Treatment of Normal Proliferating Cells With NDV

In these experiments, proliferating human PBMCs were used rather than unstimulated PBMCs, since the former cells would serve as a better comparison with proliferating tumor cells. In contrast to its enhanced cytotoxicity toward NDV-treated cancer cells, rHuTNF-α did not kill NDV-treated human PBMCs proliferating in response to Con A (table 7). Similarly, neither NDV (10 HAU), rHuTNF-α (up to 1,000 U), nor the combination of these two reagents affected the capability of normal human PBMCs during the last 18 hours to proliferate in response to Con A (data not shown). As a positive control, Act D (1 μg/mL) was added during the last 18 hours of the proliferation assay. Unlike NDV, Act D totally inhibited the uptake of ^{3}H by PBMCs (stimulation index, <1), even though it was not cytotoxic to these cells (specific ^{51}Cr release, 8%).

Discussion

In this article we investigated one aspect of the antineoplastic properties of a paramyxovirus. We focused our attention on NDV, since it has been administered in live form to cancer patients for the past 20 years with claims of beneficial results and minimal side effects (*5,9,11,12*). In our experiments, two major observations were made regarding the mechanisms by which NDV can induce lysis of tumor cells: (*a*) NDV elicits TNF-α production by human PBMCs and (*b*) TNF-α can kill NDV-treated, TNF-resistant human cancer cells but not normal proliferating human PBMCs treated with NDV. Furthermore, in marked contrast to the nonspecific effects of many cancer chemotherapeutic agents such as Act D, the combination of NDV and TNF that killed up to 67% of malignant melanoma MEL-21 cells (table 7) did not inhibit proliferation of Con A-stimulated PBMCs. These results strongly suggest an important role for TNF in some of the antineoplastic properties of NDV.

Various approaches were used to reach the conclusion that NDV is capable of inducing TNF in human PBMCs. First, it was necessary to determine if any IFN might have activity in the TNF bioassay, since NDV is a potent inducer of IFN-α (*10*). The WEHI 164 clone 13 cell line employed in the TNF bioassay was sensitive to 2 pg of TNF-α, whereas it was insensitive to 5,000 pg of the IFNs (murine IFN-α and rat IFN-γ, table 2). Supernatants from both human and rat mononuclear cells treated with NDV, but not supernatants from untreated mononuclear cells, could lyse the sensitive WEHI 164 clone 13 cells. However, these supernatants at the same dilution had no effect on the L929 cell line that is much less sensitive to the lytic effects of TNF-α. All of the lytic activity in the supernatants of NDV-treated human PBMCs could be abrogated by treatment with a specific antibody to TNF-α, but not with an antibody to TNF-β. Furthermore, three different human transformed cell lines that are resistant to the lytic effects of TNF-α could be rendered susceptible to killing by TNF-α after they were exposed to NDV.

Perhaps the most important result reported in this article is the observation that an agent (NDV) that is administered to cancer patients is a potent inducer of TNF in vitro. Levels of TNF induced by NDV reported here (400 U/10^6 PBMCs) are comparable with those induced by another paramyxovirus, Sendai virus [150 U/10^6 human PBMCs (*15*)]. Two lines of evidence also suggest that TNF is produced in vivo during viral infections. First, macrophages often predominate at sites of viral infection (*39*), and macrophages are a major cellular source of TNF-α. Second, Mak et al. (*40*) and Chapes and Tompkins (*41*) have shown that rodents infected with influenza A, Sendai, and vaccinia viruses generate macrophages that are cytotoxic toward virally infected cells but not toward uninfected cells. Since TNF-α is a potent mediator of macrophage/monocyte cytotoxicity (*42,43*), in vivo induction of TNF during viral infections seems likely.

Virus enhancement of the cytotoxic activity of TNF-α and of TNF-β occurs after exposure to vesicular stomatitis virus (*44,45*), adenovirus type 2 (*46*), and herpesvirus (*47*). Eifel et al. (*44*) postulated that this effect is due to the viral inhibition of host protein synthesis that is normally needed to overcome TNF-mediated cytotoxicity. It is well known that infection with many viruses can potently inhibit host RNA and protein synthesis (*48*). Furthermore, inhibition of protein synthesis with agents like cycloheximide confers TNF sensitivity to TNF-resistant cells (*49-51*). Strong additional evidence for a common mechanism by which viruses and protein-synthesis inhibitors augment TNF cytotoxicity is the finding that a protease inhibitor reverses the effect of both cycloheximide (*51*) and paramyxoviruses.[6] Highly relevant to our findings are the observations of Fransen et al. (*23*), who showed that a very diverse set of human cancer cells could be made dramatically more sensitive to the cytotoxicity of TNF in the presence of the RNA-synthesis inhibitor Act D. If enhancement of TNF cytotoxicity due to viral exposure occurs by the same mechanism as that induced by RNA- or protein-synthesis inhibitors, the recent work of Fransen et al. (*23*) would suggest that our observations of virus-enhanced

[6]Rood PA, Lorence RM, Kelley KW: manuscript in preparation.

TNF cytotoxicity toward MEL-14, MEL-21, and K562 cells would extend to many different types of human cancer cells. The necessity of a live virus to enhance TNF cytotoxicity toward tumor cells was demonstrated by comparing TNF cytotoxic activity toward tumor cells treated with live NDV with that toward tumor cells treated with UV radiation-inactivated NDV. The UV treatment of NDV abrogated up to 80% of TNF-α activity for NDV-treated tumor cells.[6]

An attempt to elicit in vivo production of TNF by injecting NDV into mice was unsuccessful (data not shown), presumably because an active viral infection was needed to provide a more continuous stimulus for macrophage production of TNF. NDV has not been shown to replicate in adult mice except in those with tumors (*9*). Indeed, NDV strain 73-T was first isolated after 73 passages in mouse Ehrlich ascites tumor cells (*9*), which probably enhanced its tumor specificity and reduced its neurovirulence. Therefore, a logical extension of our results would be to test for possible synergistic effects when both NDV strain 73-T and TNF-α are administered in vivo to tumor-bearing rodents.

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DEFENSE MECHANISM AGAINST NEWCASTLE DISEASE VIRUS IN CHICKEN MAXILLARY SINUS*

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Introduction

Chronic paranasal sinusitis still remains one of the most prevalent diseases of the nose and accessory sinuses in Japan[1], although the incidence of nasal allugy recently tends to be taking the place of sinusitis[2].

Whatever the original function of the paranasal sinuses may have been, the paranasal sinuses are directly continuous portions of the upper respiratory tract and thus often is affected in infections of the latter. There are no microorganisms which have a special affinity for sinus infection, although the sinuses are often the site of a prolonged residue of what earlier was a more generalized respiratory infection. Why this is so is not clear, but several factors listed below may be of etiologic significance; impaired mucociliary activity[3-5], nutritional disorders[4], and anatomic or physiologic features[6,7] which one way or another obstruct free drainage from the sinuses, such as allergic polyps[8]. On the other hand, the etiologic factors of primary malignant neoplasms of the maxillary sinus are difficult to identify, but many of these patients have a long history of chronic sinusitis, and metaplasia of the ciliated epithelium to the squamous type in the sinus plays a significant role in the development of these tumors[9-11].

Thus, it is highly important to investigate the cause of maxillary sinusitis.

The mucociliary system constitutes an effective protective mechanism against inhaled materials and infectious microorganisms; its failure is often implicated in respiratory tract infections[12-15]. Bang and co-workers[28] found that there was a great increase in the number of infected cells if a chicken is first given intranasal cocain so that cilia are paralyzed.

There are many papers concerning experimental viral infection by intranasal inoculation[16-28], especially cases with regard to aspects of morphology[20], physiology[21,22], pathology[23], mucociliary transport[24,25], cell labeling index[26], and the lymphoid system[27]. Responses to viral infections of chicken nasal tissues[28] have been studied in Dr. Bang's laboratory for twenty years. From these experiments, Bang and Bang concluded that

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Newcastle disease virus usually does not infect the maxillary sinus in chickens.

The purpose of this study was to investigate the mechanism of infection in the maxillary sinus and turbinate by intranasal infection.

Materials and Methods

Chickens. One day old Babcock chickens were obtained from a commercial source for all experiments and they were maintained on a standard commercial mash chicken diet until ready for experimental use, for the most part at the age of 21 days. Water and food were available *ad libitum* throughout.

Virus. Undiluted mesogenic Newcastle disease virus B strain (NDV-B) was from a frozen stock preserved from the 12th allantoic fluid passage and had a titer of 7×10^8 plaque forming unit (PFU)/ml and a hemagglutination (HA) titer of 1,280. 0.01 ml for the naris or 0.005 ml for the maxillary sinus through the thin membranous palate were inoculated in this study; each chicken was subsequently checked for virus during the disease or for antibody (HAI) subsequent to infection. All inoculated chickens showed positive histologic evidence of infection.

Virus isolation and titration of serum HAI antibody. Under a dissecting microscope, the turbinate was removed; then turbinates were placed in 0.9 ml of phosphate buffered solution containing 100 units of penicillin and streptomycin. After homogenization with a grinder, the sample was used for virus quantitation as 1% homogenate containing NDV. The maxillary sinus swabs were done with single Q-tip applicators, which were then placed in 1 ml of buffered solution containing 100 units of penicillin and streptomycin. Serial 10 fold dilution of each sample obtained from sinus swabs or homogenated tissues were tested for virus isolation by the plaque method. When an experiment was terminated, chickens were killed by cardiac bleeding. Using a microtiter kit, hemagglutinin inhibition titrations were performed on days 1, 3, 5, 7, and 9 after inoculation.

Plaque method. Plaque assay was done as follows[80]; ten-day-old chick embryos were used to prepare monolayer cultures of fibroblasts, containing about 2 million cells per petri dish (60× 60 mm). The medium used for maintaining the cells consisted of 10% calf serum and 90% modified Eagle's medium containing 100 units of penicillin and streptomycin/ml. After cultured for 48 hours at 37°C in the presence of 5% CO_2, the cells were washed twice with 4 ml of modified Eagle's medium, inoculated with 0.2 ml of inoculum which were adsorbed for 1 hour at 37°C, and then overlayed with 6 ml of the same culture medium containing 1% methylcellulose. After incubation with 5% CO_2 for 4 days at 37°C plates were washed with phosphate buffered solution and stained with 2.5% crystal violet. The number of plaques present at this time was determined on two plates, each of which received 0.2 ml of ten-fold inoculum.

Mucociliary clearance. The time required for mucociliary clearance of turbinates and sinuses was measured; in the text, these are designated as turbinate clearance time and sinus clearance time. The method has been described in detail by Wakabayashi et al.[14] and Ukai et al.[13] Briefly, the chicken's body was held in the plastic holder and its mouth was held open with an adjustable mouth opener. To measure mucociliary clearance time, the entire apparatus was turned upside down and movement of mucus on the postnasal fossa through the palatine cleft was observed with a stereoscopic microscope, which has a bright cool spotlight, at ten-fold magnification. To measure turbinate time, one drop (10–13 μl) of nontoxic McCormic green food dye was placed in the each naris, and the time from this movement until dye appeared at the choanal margin was measured with a stopwatch. To measure sinus clearance time, 3 μl of the dye was injected through the thin membranous palate in the same way. If the dye had not appeared in 6 minutes, the time was recorded as 6 minutes.

Histologic studies. In selected experiments, chickens killed by cardiac bleeding were decapitated and heads were fixed in 10% neutral buffered formalin, decalcified in 2% nitric acid, and neutralized

in 5% sodium sulfate. Six micron sections were stained in Hematoxylin and eosin and/or the combined Alcian blue periodic acid Schiff stain with the Alcian blue at 1%, pH 1.0.

Experimental procedures. Chickens infected NDV were divided into three groups; (1) those inoculated intranasally, (2) those inoculated intrasinusly, and (3) those treated intrasinusly with 5% cocain at 24 hours after intranasal inoculation of NDV.

Results

1) *Effects of intranasal inoculation with NDV-B.* The most remarkable characteristic induced by NDV infection following intranasal inoculation was the destruction of significant portions of the inner surface of the turbinate and septum (Fig. 1). Sinus lesions though sometimes present in NDV infection were not common (Fig. 2). Those findings were expected to alter mucociliary clearance times in the turbinate but not in the sinus.

Slowing of the turbinate times became gradually more marked between the third and fifth days, at which time activity resumes as long as nine days after inoculation. Although the changes in turbinate time caused by NDV infection varied considerably in individual chickens, the effects were usually moderate. Sinus time was not significantly affected by NDV infection (Fig. 3).

The question whether virus recovery was obtained from the maxillary sinus of individual chickens which were infected by the intranasal inoculation was tested. As Fig. 4 shows, there was a great difference between the amount of virus in the turbinate and maxillary

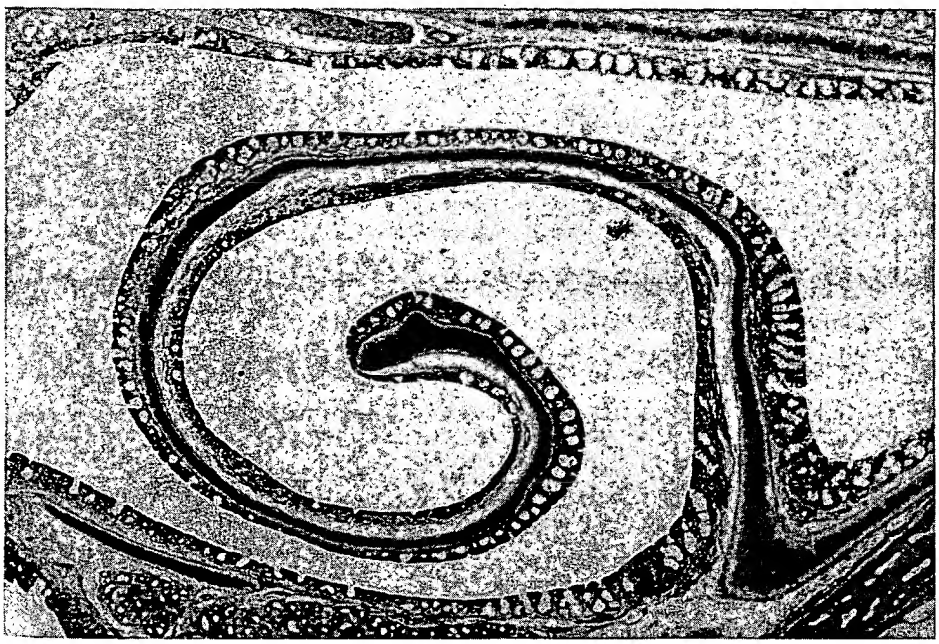

Fig. 1. Turbinate from 3-week-old chicken infected with NDV intranasally for 3 days. The inner surface of the turbinate scroll is selectively destructed. (H & E, ×40).

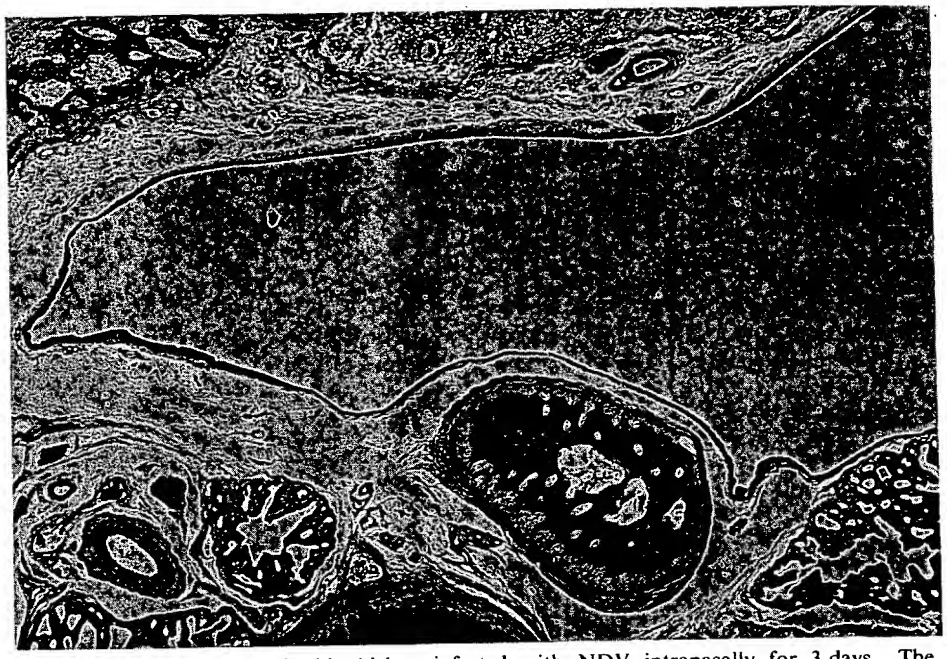

Fig. 2. Sinus from 3-week-old chicken infected with NDV intranasally for 3 days. The maxillary sinus is almost normal (H & E, ×40).

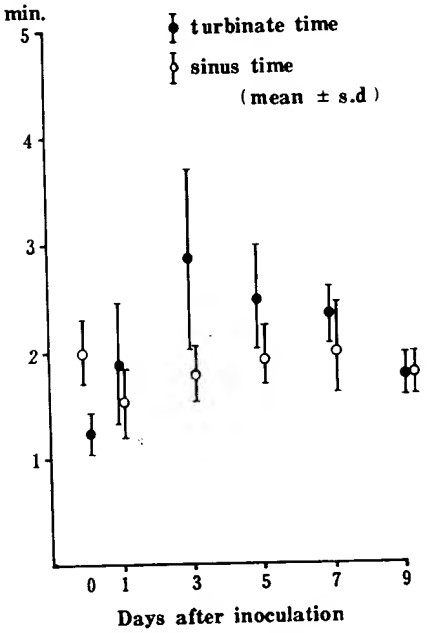

Fig. 3. Mucociliary transport time in chickens after intranasal inoculation with NDV.

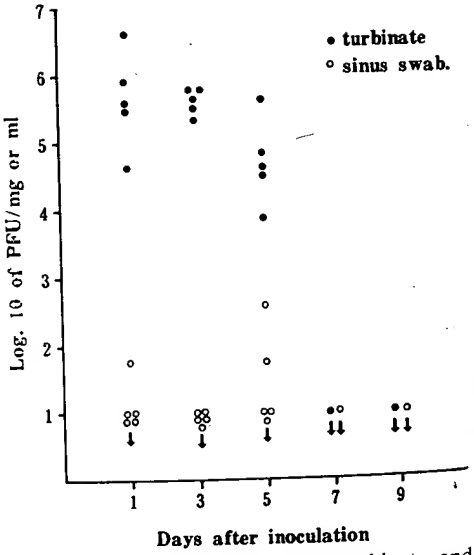

Fig. 4. Amount of virus in the turbinate and maxillary sinus of the chicken after intranasal inoculation with NDV.

sinus. Good virus recovery was obtained from the nasal turbinate of all animals on days 1 through 5 but never beyond 7. There was no virus recovery from the sinus swab up to nine days after inoculation, and only small amounts were found in one out of five chickens for day 1, and two out of five chickens for day 5.

2) *Effects of intrasinus inoculation with NDV–B.* Inoculation into the maxillary sinus through the palate with 5 μl of undiluted NDV–B produced acute destruction of maxillary sinus mucosae in most of the chickens. Some of the chickens showed loss of cilia, infiltration of polymorphonuclear and mononuclear cells, and/or cystic formation and destruction of sinus epithelium. Furthermore giant cells formation were sporadically found (Fig. 5). There was destruction of the inner and outer surface of the turbinate in several chickens.

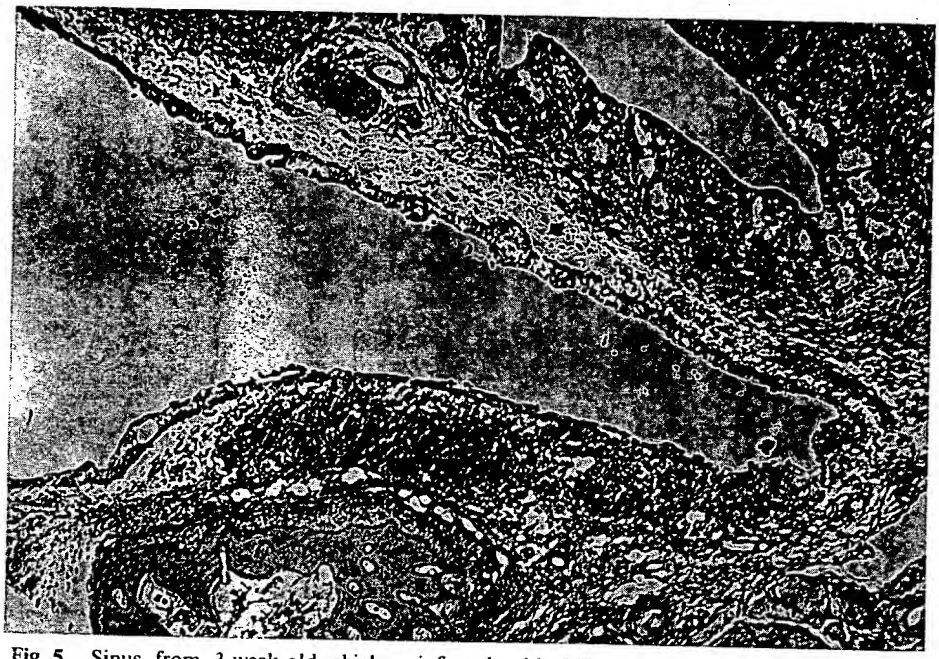

Fig. 5. Sinus from 3-week-old chicken infected with NDV intrasinusly for 3 days. The maxillary sinus is infected with NDV, showing loss of cilia, infiltration of polymorphonuclear and lymphocytic cells (H & E, ×100).

Mucociliary transport time in the sinus showed significant slowing on days 3 or 5 after intrasinus inoculation. Changes in turbinate clearance times were mild (Fig. 6).

A high titer of virus was obtained from the turbinate tissue and sinus swabs of all 5 chickens on days 1 through 5 but the titer had dropped on day 7 and 9 (Fig. 7).

3) *Effects of the treatment with 5% cocain on the maxillary sinus following intranasal inoculation with NDV–B.* We investigated the effect of 5% cocain on sinus clearance times.

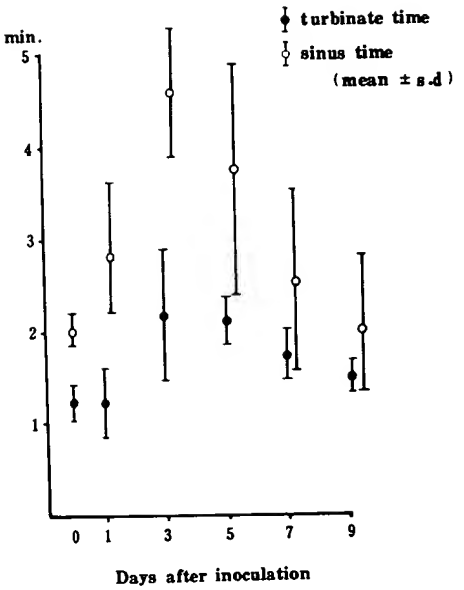

Fig. 6. Mucociliary transport time in chickens after intrasinus inoculation with NDV.

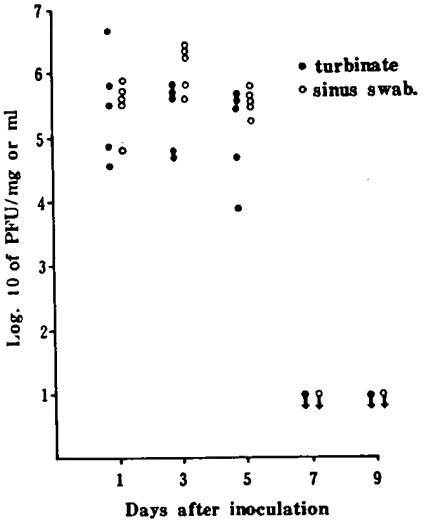

Fig. 7. Amount of virus in the turbinate and maxillary sinus of chickens after intrasinus inoculation with NDV.

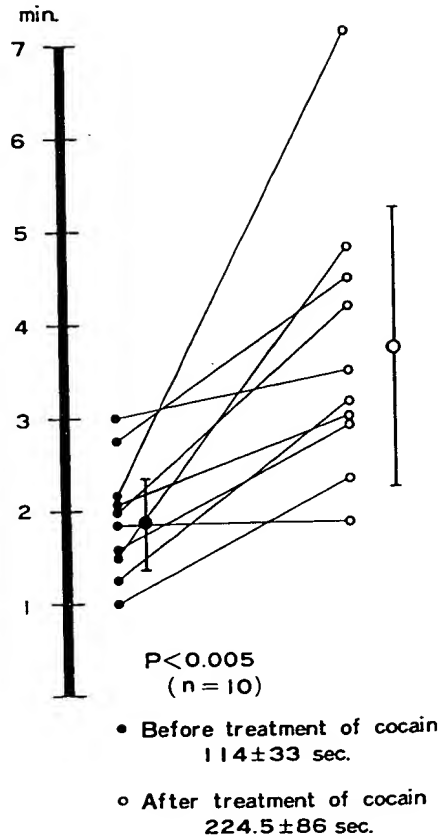

Fig. 8. Effects of the treatment with cocain on mucociliary transport time in the maxillary sinus.

Fig. 10.

Mucocilia
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clearance
and return

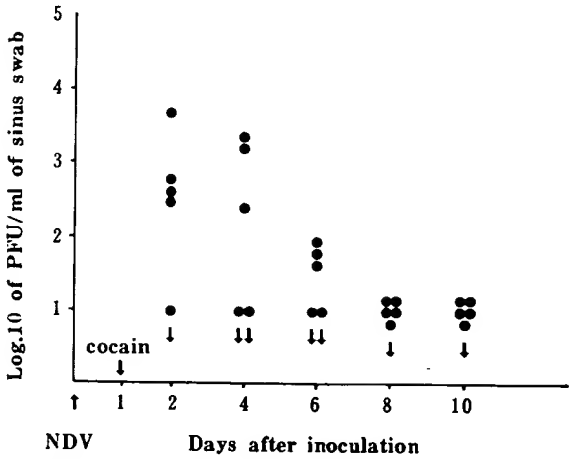

Fig. 9. Amount of virus from sinuses which were treated with cocain after 24 hours of intranasal inoculation of NDV.

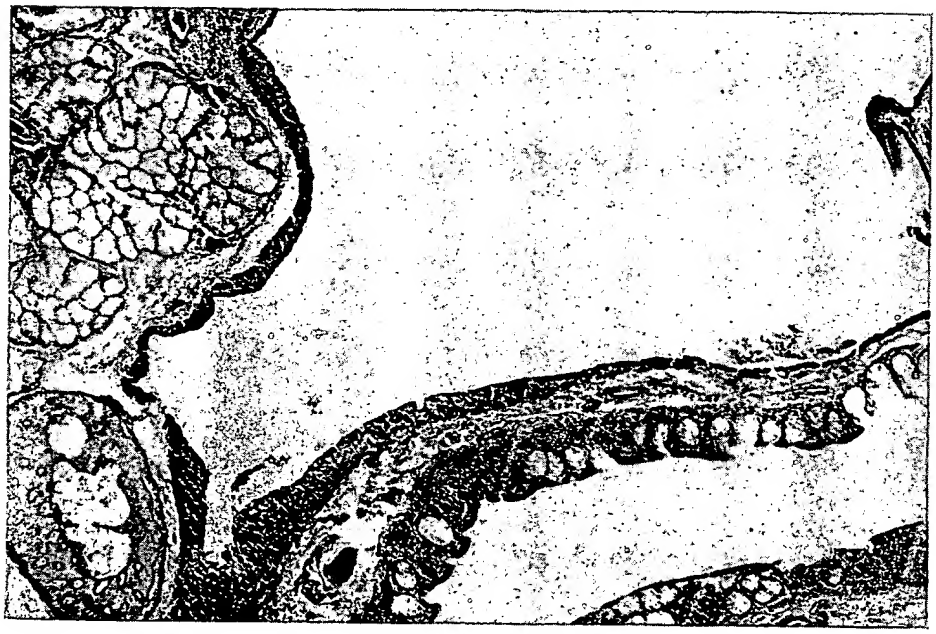

Fig. 10. Six day infection; sinus treated with cocain after 24 hours of intranasal inoculation with NDV. The maxillary sinus seems to be infected with NDV, showing lymphocytic infiltration (H & E, ×40).

Mucociliary transport time in the maxillary sinus was measured at 10 minutes after instillation of 0.05 ml of 5% cocain into the sinus. As Fig. 8 shows, 5% cocain slowed sinus clearance time significantly ($n=10$, $p<0.005$, paired t-test), lasting more than 30 minutes and returned to normal one hour after the instillation of cocain. The histologic examination

showed nearly all cilia of the sinus mucosae were intact.

The amount of virus was measured from sinus swabs of individual chickens which were treated with 0.05 ml of cocain after 24 hours of intranasal inoculation with NDV-B. Virus was recovered from 4 of 5 chickens on day 2, and from 3 of 5 chickens on day 4 and 6 but never beyond day 8 after infection (Fig. 9). This suggests the prevalence of virus through the narrow ostium into the maxillary sinus by the slowing of mucociliary clearance due to direct paralysis of the cilia of sinus mucosae.

Examination of histologic sections for effect of 5% cocain on intranasal inoculation with NDV showed inflammatory changes such as lymphocytic and polymorphonuclear infiltration and/or destruction of ciliated cells in sinus epithelium in all 5 chickens.

Discussion

The chicken nasal and paranasal sinus mucous membranes have proved to be excellent models to study defense mechanisms against maxillary sinus infection. We suggest that intranasal NDV infection of maxillary sinus may be prevented by the mucociliary activity in the maxillary sinus, especially in the sinus ostium.

The chicken was selected because its nasal structure is similar to that of humans, i.e., relatively simple turbinates, true sinuses and a mucociliated, acinar, respiratory epithelium[29].

The lesions induced by NDV following intranasal inoculation usually produced selective destruction of significant portions of the inner surface of the turbinate scroll, but sinus lesions though sometimes present in NDV infection were not common (Figs. 1 and 2). These results were supported by the amount of virus and mucociliary transport time in the turbinate and sinus (Figs. 3 and 4).

There are five major possible explanations for a reason why the chicken maxillary sinus usually was not infected by intranasal NDV-B instillation; (a) tissue specificity against NDV-B; (b) selective local immune responses against NDV-B; (c) mucociliary activity and direction of mucociliary flow; (d) anatomical architecture of the sinus ostium; (e) virulence of the virus. However, the chicken maxillary sinus was infected by the intrasinus inoculation with NDV-B (Figs. 5, 6 and 7) and also by the treatment of 5% cocain in the maxillary sinus at 24 hours after intranasal inoculation with NDV-B (Figs. 9 and 10). Therefore sinus infection due to intranasal inoculation with NDV-B may have been prevented by the mucociliary activity and/or direction of the mucociliary flow in the maxillary sinus.

Dye inoculated into the naris reaches the tip of the turbinate almost immediately. It moves first over the inner of the middle turbinate, then removes in a straight line into the posterior nasal fossa and choana[31]. This explains the localization of infection in the turbinate by intranasal infection.

Once the dye is injected into the maxillary sinus through the palate, it first spreads over the floor of the sinus, then is carried by mucociliary action to the sinus ostium where it fans

out and moves directly into the posterior nasal fossa[14]. One assumes that the protective effect of the sinus by intranasal NDV inoculation is also related to the route of mucociliary flow in the sinus.

On the other hand, in order to produce infection, a virus must penetrate the mucous blanket against mucociliary clearance and have access to a susceptible cell. Ciliary paralysis increased the number of infected cells by 10 to 50 times over the control value[28]. Thus, mucociliary activity is one of the most important factors for the defense mechanism of infection.

Depression of nasal mucociliary activity in intact animals by local application of cocain has been demonstrated[28,32-34]. We studied the effect of cocain on sinus clearance time. Although cessation of the mucociliary function was not observed by the application of 5% cocain into the maxillary sinus, deceleration of sinus clearance time after treatment was statistically significant ($p<0.005$, Fig. 8). Nevertheless, the virus was recovered from the chicken maxillary sinuses which were treated with 5% cocain after 24 hours of intranasal inoculation (Fig. 9), and this was supported by histologic examination (Fig. 10). From these results, the mucociliary activity in the maxillary sinus, especially in the sinus ostium, rather than the direction of the mucociliary flow may be the most important factor in protecting against sinus infection.

Mucociliary clearance rates vary in different parts of the nose, as do the depth and mucous composition of acini[23,25]. Mucociliary actions transport fluids produced by mucous glands and goblet cells. The circumference of the transporting route decreases rapidly from the chicken sinus cavity to the sinus ostium. This convergence of mucous layers would fill the sinus unless the fluid was being removed by the many-fold increased flow. We believe that the defense mechanisms against sinus infection may be explained by the acceleration of the mucociliary transport rate in the sinus and/or sinus ostium which we will refer to as mucociliary homeostasis, that is, a continuously interacting localized adjustment of secretory components in maintaining a smooth flow of the mucociliary blanket.

Such a homeostatic mechanism, regulated in a way not yet fully understood, could be due to an effect of the nasal infection on stimulating secretion within the ostial gland. In the chicken, the acinar glands surrounding the ostia are conspicuously deeper, and differ histochemically, from those elsewhere in the fossa[25]. In developing human embryo the acinar glands intrinsic to the maxillary sinus are also clearly different structurely from those of the main fossa[36].

Although normal nasal mucociliary flow rates in any individual depend on the ciliary rates plus secretion of mucus of a particular viscosity, the direction and the rate of the ciliary beat may be affected by changes in the quality and quantity of mucus[37]. Phipps and Richardson[38] reported that mechanical stimulation of the nose and nasopharynx increased tracheal mucus output by nerve reflexes. Ukai et al.[39] also found that mechanical stimulation on the chicken palatine cleft accelerated the rate of nasal mucociliary clearance.

Thus, there may be an important relationship between the increased mucus output and the accelerated mucociliary clearance. Intranasal mucociliary defense mechanisms may be explained by the acceleration of the mucociliary clearance due to the secretion of mucus of a particular viscosity.

Summary

The mechanism of resistance to mesogenic Newcastle disease virus infection in the chicken maxillary sinus and turbinate by intranasal inoculation was studied.

The lesions induced by NDV-B following intranasal inoculation usually produced a selective destruction of significant portions of the inner surface of the turbinate scroll, but sinus lesions though sometimes present in NDV infection were not common. These results were supported by the amount of virus and the mucociliary transport time in turbinate and sinus.

The chicken maxillary sinus was infected by intrasinus inoculation with NDV-B. Although cessation of the mucociliary function was not observed by the application of 5% cocain into the maxillary sinus, the deceleration of the sinus clearance time after treatment was statistically significant. Virus was recovered from the chicken maxillary sinuses which were treated with 5% cocain after 24 hours of intranasal NDV infection, and this is supported by the histologic examinations.

From these results, mucociliary activity in the maxillary sinus, especially in the sinus ostium, rather than the direction of the mucociliary flow, is one of the most important factors in protecting against sinus infection.

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